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Changes in oleuropein levels during differentiation and development of floral buds in 'Arbequina' olives

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Abstract

Oleuropein is the most abundant biologically active phenolic compound in olives. It has been extensively studied for human health benefits but its role in plant development processes has received limited attention. Changes in the levels of oleuropein during early stages of flower formation and during fruit development and maturation were determined using high performance liquid chromatography. Oleuropein and other phenolic compounds were identified by comparing retention time and UV spectra with standard compounds using photodiode array detectors. Quantitative measurements were based on peak areas relative to standards. Oleuropein levels sharply decreased during the transition from vegetative to flower buds, consistent with earlier reports that higher levels of exogenously applied oleuropein inhibited flowering in *Kalanchoe blossfeldiana*. Oleuropein levels rapidly increased with the expansion of fertilized pistils and then sharply declined with fruit maturity. There was only a modest decline in oleuropein levels between immature and fully expanded leaves. Hesperidin, which occurs in relatively small amounts, also declined considerably during early floral buds formation. Maximum levels of verbascoside were found in fully developed green fruits while maximum levels of luteolin-7-*O*-glucoside and luteolin-4-*O*-glucoside were found in fully expanded leaves.
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Keywords: Oleuropein; Arbequina; Olives; Phenolics; Flowering; Fruiting

1. Introduction

Oleuropein is the most abundant phenolic compound in olive leaves and fruits and is responsible for the characteristic bitterness of olive fruit (Andrews et al., 2003; Soler-Rivas et al., 2000). Health benefits of this compound have been extensively investigated. It has been reported that oleuropein, and related compounds such as tyrosol, verbascoside, ligustroside, and demethyoleuropein, act as antioxidants and lower the risk of coronary diseases (Manna et al., 2002; Visioli et al., 1998; Wiseman et al., 1996), several cancers (Owen et al., 2000; Tripoli et al., 2005), and could have antimicrobial and anti viral activity (Bisingnano et al., 1999; Federici and Bongi, 1983; Fleming et al., 1973). In addition, oleuropein has been reported to repel insects (Lo Scalzo et al., 1994) and protect against pathogens (Uccella, 2001).

The role of oleuropein in the developmental processes of olive trees has received limited attention, although changes in oleuropein levels during fruit development and maturation (Amiot et al., 1986, 1989; Ryan et al., 1999, 2003), and during Spanish-style processing of green olive fruits have been reported (Brenes et al., 1995). In one study, oleuropein was shown to inhibit the growth of potted olive trees and markedly decrease olive callus growth, induce irreversible stomatal closure in *Commelina communis*, and stimulate rooting in *Vinga radiata* (Bongi, 1986). Others have noted that phenolic compounds in olives (e.g., chlorogenic acid) affected rooting, callus growth, and flowering (Lavee et al., 1994; Lavee and Avidan, 1982; Ozkaya and Celik, 1999).

Oleuropein is also found in several plant species other than *Olea europaea* (olive) (Soler-Rivas et al., 2000). Exogenous application of oleuropein inhibited flowering (at doses lower than 10^{-4} M) in *Kalanchoe blossfeldiana* (Bongi, 1986). It is therefore conceivable that as a major phenolic compound, oleuropein could play a role in flowering in olives. This study examined the changes in oleuropein levels, in 'Arbequina' olives during the differentiation of lateral vegetative buds into flowering buds and the subsequent growth and development of floral structures.

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2. Materials and methods

2.1. Plant samples

Lateral vegetative buds were obtained from 4-year-old 'Arbequina' trees (10 replicate tree in each treatment) in late February 2005 that were kept either under non inducing conditions in a greenhouse (i.e., 26 ± 1 °C nighttime and 28 ± 1 °C daytime temperature) or outside under the ambient temperature of South Texas (Weslaco) where olives do not flower. Flower buds were obtained from a set of trees kept in a locally designed growth chamber (Malik and Bradford, 2005a) under flower inducing conditions from mid November 2004 until 14 February 2005; i.e., 8 ± 1 °C nighttime temperatures that progressively increased during day to reach 18 ± 1 °C for 1–2 h followed by a progressive decline. From mid-February nighttime temperature in growth chamber was progressively increased to 12 ± 1 °C to initiate flower bud formation; we have shown extensive flowering in 'Arbequina' olives under these conditions (Malik and Bradford, 2005b). The remaining buds on trees under non-inducing conditions did not produce flowers but the remaining buds on the trees under inducing conditions flowered heavily. At the time of excision, the tiny lateral buds (1–2 mm long) had already taken the shape of floral buds, broader than the acute elliptical shape of vegetative buds (1–1.5 mm). Several hundred vegetative or flowering buds were excised from the axis of leaves, with sharp pointed forceps, from different branches of different trees (there were 10 trees under each set of conditions) to obtain at least 1–2 g of fresh tissue. Later samples included, complete open flowers, staminate flowers, 1–2 mm diameter fruits that had just shed petals and other floral structures, and 5–7 mm diameter fruits. Fully developed green and dark brown or black fruits were obtained from 'Arbequina' trees growing in Carrizo Springs, TX. Immature leaves (5–7 mm long) and fully expanded leaves were collected from trees in the open field in Weslaco. All samples were immediately frozen and stored at −80 °C until used for analyses; stones were removed from fully developed green and black fruits and only pulp was frozen for analyses.

2.2. Sample preparation and extraction

Frozen plant samples were pulverized in liquid nitrogen as described previously, and stored in 15 ml polyethylene plastic screw cap tubes at −80 °C (Malik and Bradford, 2005c). An aliquot of 250 mg powdered tissue (including gallic acid as internal standard) was extracted in 10 ml of 80% methanol by blending with a polytron (Brinkman model PT 3100) at maximum speed for 30 s (three times at 10 s intervals). The mixture was centrifuged at 39,000 × *g* for 20 min, and after removing the supernatant liquid the pellet was re-extracted with 10 ml of 80% methanol. The two extracts were combined and the volume adjusted to 25 ml. The extract was passed through a 20 μ filter, and aliquots (20 μl) from the filtered extract were directly analyzed by high performance liquid chromatography (HPLC).

2.3. High performance liquid chromatography

HPLC analyses were performed with a Waters (Milford MA) Alliance HPLC system (model 2695) equipped with photodiode array detector (model 2996). Oleuropein and other phenolic compounds in olive tissue were separated by reverse phase HPLC using a Waters Symmetry C_{18} (5 μm particle size) column (3.9 mm × 150 mm) maintained at 35 °C during chromatographic runs. The column was eluted at a flow rate of 1 ml/min with a gradient of solvent system comprising of 100% acetonitrile (solvent A) and 0.02% trifluoroacetic acid in water (solvent B). Initially the gradient comprised of 5% A and 95% B, then the solvent A was linearly increased to 10% in 10 min. After 10 min solvent A was linearly increased to 30% in 24 min, and thereafter, solvent A was increased to 40% in 11 min followed by ramping to 80% A in 10 min. The column was equilibrated with 95% B for 10 min between each run. A 20 μl aliquot of extract or standard solution was injected for each run and elution profiles were detected at 280 nm using photodiode detectors. Oleuropein and other phenolic compounds in the extract were identified by matching retention time and the UV spectra of a peak in the extract chromatogram with the peak of a known standard compound. For quantitative measurements of oleuropein, or any other phenolic compound, a regression curve was developed by injecting different amounts of a known standard compound in the HPLC column.

2.4. Total phenols

The total phenol content was determined colorimetrically at 765 nm, using Folin-Ciocalteu reagent (Gutfinger, 1981). A 50 μl aliquot of extract was combined with 50 μl of Folins-Ciocalteu reagent, 150 μl of 20% (w/v) sodium carbonate and 1370 μl of Milli-Q water. Pure oleuropein was used to develop standard curves. A Beckman-Coulter UV–vis Spectrophotometer (model DU530) was used to measure optical density.

2.5. Statistical analysis

Samples were randomly collected from 10 replicate trees in each treatment. A minimum of three replicate extractions were performed from each treatment followed by a minimum of three replicate HPLC analyses from each extracts. Statistical analyses were conducted on treatment means using the *t*-test procedure of InStat® software, Version 3.0, (GraphPad, 5755 Oberlin Drive, San Diego, CA).

3. Results and discussion

3.1. Oleuropein levels during various floral and fruit developmental stages

Oleuropein levels in buds and fruits of 'Arbequina' olives were within the range of levels reported for Manzanillo olives (Table 1; Ryan et al., 1999). Changes in oleuropein levels during fruit development of olive cultivars other than 'Arbequina' have been reported in the past (Amiot et al.,

Table 1
Oleuropein and total phenol content in vegetative and flowering buds and in developing and mature fruits of 'Arbequina' olives

Type of sample	Oleuropein (mg/g) fresh weight	Total phenols (mg/g) fresh weight	Oleuropein as % of vegetative buds	Total phenols as % vegetative buds
Vegetative buds	58.36 ± 1.74	265.48 ± 4.39	100	100
Flowering buds	15.70 ± 0.92	109.05 ± 3.71	27	41
Flowers (complete)	20.99 ± 0.15	63.67 ± 2.53	36	24
Flowers (staminate)	15.32 ± 0.24	41.74 ± 0.95	26	16
Fruit stage 1 (2–3 mm diameter)	50.82 ± 1.88	121.79 ± 3.18	87	46
Fruit stage 2 (5–7 mm diameter)	40.07 ± 1.96	69.76 ± 3.47	69	26
Fruit mature green (10–13 mm diameter)	13.65 ± 0.48	57.58 ± 2.17	23	22
Fruit mature black (10–13 mm diameter)	0.0 ± 0.0	48.51 ± 2.10	0	17

1989; Ryan et al., 1999), but to our knowledge this is the first time changes in oleuropein levels at very early stages of the transition from vegetative to floral buds have been studied. Drastic reduction in oleuropein levels was observed in floral buds (1–2 mm) compared to vegetative buds (Table 1). Since the vegetative buds were taken from trees kept in the greenhouse at fairly warm temperatures (26–29 °C), we also studied oleuropein levels in vegetative buds of the outside trees in Weslaco, TX (where olives normally do not flower) that received 100–200 h of chilling temperatures during November 2004–February 2005. The oleuropein levels in field grown vegetative buds were 54.04 ± 1.81 mg g^{-1} compared to 58.36 ± 1.74 mg g in vegetative buds from greenhouse grown trees. Thus, there was very small difference (about 11%; insignificant at $P < 0.01$) in oleuropein between vegetative buds of trees kept at different temperature regimes. However, there was a 73% reduction in oleuropein levels (significant at $P < 0.0001$) in floral buds indicating that observed changes in oleuropein levels in floral buds were developmentally regulated. These results are consistent with the finding of Bongi (1986) that exogenous application of oleuropein in *K. blossfeldiana* (i.e., increasing the levels of oleuropein) inhibited flowering. A decline in oleuropein levels was also noted when vegetative buds sprouted into leaves but the decline was far less pronounced compared to the decline in floral buds (Tables 1 and 2). It would be interesting to determine if similar dramatic changes in oleuropein levels occur during floral differentiation in other olive cultivars. Total phenolic compounds were also reduced but changes were less than changes in oleuropein; i.e., 59% reduction in total phenols versus 73% reduction in oleuropein levels.

After floral bud differentiation, oleuropein levels progressively increased from the flowering to fruiting stage and then declined as the fruit began to mature, reaching to negligible levels in the fully mature black fruits (Table 1). Changes in oleuropein levels during fruit development and maturity in 'Arbequina' show a similar pattern reported for other cultivars (Amiot et al., 1986; Ryan et al., 1999). It appears that the rise in oleuropein levels in floral structures is associated with growth activity in the pistil. This is supported by the fact that oleuropein levels were 27% lower in staminate flowers and also because oleuropein levels climaxed in stage 1 fruits (2 mm in diameter) that were basically actively growing pistils without other floral structures; i.e., 50.82 ± 1.88 mg g^{-1} in 2 mm fruits compared to 15.70 ± 0.92 mg g^{-1} in flowering buds (Table 1). Although the difference in oleuropein levels between complete and staminate flowers was not as dramatic as that between floral and vegetative buds, but still it was a significant difference at $P < 0.0001$.

From stage 1 fruits (2 mm diameter fruits) there was a 21% reduction in oleuropein levels in still expanding stage 2 fruits

Table 2
Quantitative estimates of major phenolic compounds found in various olive tissues

Type of sample	Fresh weight (mg/g)					
	Luteolin-7-*O*-glucoside	Verbascoside	Luteolin-4-*O*-glucoside	Hesperidin	Rutin	Chlorogenic acid
Vegetative buds	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	3.86 ± 0.08	+[a]	+
Flowering buds	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.58 ± 0.04	+	+
Flowers (staminate)	0.0 ± 0.0	1.37 ± 0.03	0.0 ± 0.0	0.0 ± 0.0	+	−[b]
Flowers (complete)	0.0 ± 0.0	2.76 ± 0.06	0.0 ± 0.0	0.0 ± 0.0	+	−
Fruit stage 1 (2–3 mm diameter)	0.09 ± 0.01	0.79 ± 0.03	0.0 ± 0.0	0.0 ± 0.0	+	−
Fruit stage 2 (5–7 mm diameter)	0.04 ± 0.01	0.77 ± 0.01	0.0 ± 0.0	0.0 ± 0.0	+	−
Fruit mature green (10–13 mm diameter)	0.83 ± 0.02	3.81 ± 0.09	0.0 ± 0.0	0.0 ± 0.0	0.54 ± .02	−
Fruit mature black (10–13 mm diameter)	0.53 ± 0.02	2.82 ± 0.02	0.0 ± 0.0	0.0 ± 0.0	0.54 ± .02	−
Immature leaf[c]	0.16 ± 0.01	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	−	−
Mature leaf[d]	1.81 ± 0.06	0.66 ± 0.02	0.83 ± 0.01	0.0 ± 0.0	−	−

[a] + indicates levels detectable but below quantitation.
[b] − indicates levels below detection.
[c] Oleuropein 38.13 ± 1.81 mg g^{-1}.
[d] Oleuropein 34.07 ± 0.87 mg g^{-1}.

(5–7 mm diameter). A significant reduction in oleuropein levels (73% compared to 2 mm fruits) in the flesh of fully developed light green fruits (the fruits were still firm) is consistent with earlier reports of increased hydrolytic enzymes activity during early maturation (Briante et al., 2002). Of course, the hydrolytic activity reaches a maximum during ripening of black olives. We also observed a sharp decline in oleuropein levels in black olives.

The oleuropein levels in immature leaves (5–7 mm long) was 38.13 ± 1.81 mg g^{-1} but remained fairly constant throughout the full expansion of the leaf when 34.07 ± 0.87 mg g^{-1} of oleuropein was found in fully expanded leaves (Table 2). Since the oleuropein levels did not increase in expanding leaves, it indicates the rapid rise in oleuropein from flower buds to stage 1 fruits was specifically related to the growth activity of pistils and not due to general tissue growth processes.

3.2. Levels of other phenolic compounds found in different olive tissue

Although this study was initiated primarily to study changes in oleuropein levels during floral development, data on the levels of a few other phenolic compounds identified during the study are given in Table 2. Hesperidin was found in much smaller quantities relative to oleuropein in vegetative and flowering buds, but percentage wise its levels also reduced sharply during floral differentiation; i.e., 85% less in floral buds compared to vegetative buds (Table 2). In addition, rutin and chlorogenic acid were also detected in vegetative and flowering buds, but with our extraction and separation technique their levels were not enough for accurate quantitative measurements. Luteolin-7-glucoside was below detection in buds and flowers but was present in developing and mature fruits and leaves, its levels were highest in mature leaves (Table 2). Quantitative levels of luteolin-4-glucoside were found only in mature leaves. Verbascoside was present in measurable levels in all tissue except buds and immature leaves, and its levels were highest in fully mature green fruits and then declined 25% in black olives (Table 2). Since the rise of verbascoside in fully developed fruits coincides with decline in oleuropein in mature fruits and leaves (Tables 1 and 2), although not to the same extent, it is possible that a part of oleuropein degradation during maturation might contribute to rise in verbascoside. For example, it has been noted that degradation of oleuropein could produce hydroxytyrosol (Brenes et al., 1995; Romani et al., 1999), which then can be converted into verbascoside (Ryan et al., 2003). Apparently, levels of luteolin-7-glucoside, luteolin-4-glucoside, and verbascoside do not seem to rise or decline parallel with transition of vegetative buds to floral buds or early flower development stages. The highest levels of luteolin-7-glucoside and luteolin-4-glucoside were found in mature leaves.

4. Conclusion

Oleuropein levels sharply declined during the transition from vegetative to flowering in the buds of 'Arbequina' olives. This is an important finding in the light of the fact that exogenous application of oleuropein was reported to inhibit flowering in *K. blossfeldiana.* Apparently, oleuropein levels began to increase with the expansion of fertilized pistils and rapidly declined when fruits matured. Further investigations of the role oleuropein in the regulation of flowering and fruit development in olives are needed. It is also not clear when and how oleuropein levels accumulated maximally in vegetative buds. In addition to oleuropein, hesperidin levels (that occurs at much smaller amounts compared to oleuropein) also decrease extensively during transition from vegetative buds to flowering buds. Percentage wise the decline in total phenols was smaller than decrease in oleuropein or hesperidin. The present study could not establish any correlation between other phenolic compounds with transition of vegetative buds to flowering buds. Maximum levels of verbascoside were found in mature green fruits while maximal levels of luteolin-7-*O*-glucoside and luteolin-4-*O*-glucoside were found in mature leaves.

References

Amiot, M.-J., Fleuriet, A., Macheix, J.-J., 1986. Importance and evolution of phenolic compounds in olive during growth and maturation. J. Agric. Food Chem. 34, 823–826.

Amiot, M.-J., Fleuriet, A., Macheix, J.-J., 1989. Accumulation of oleuropein derivatives during olive maturation. Phytochemistry 28, 67–69.

Andrews, P., Busch, J.L.H.C., Joode, T.D., Groenewegen, A., Alexandre, H., 2003. Sensory properties of virgin olive oil polyphenols: identification of deacetoxy-ligstroside agglycon as a key contributor to pungency. J. Agric. Food Chem. 51, 1415–1420.

Bisingnano, G., Tomaino, A., Lo Cascio, R., Crisafi, G., Uccella, N., Saija, A., 1999. On the in vitro antimicrobial activity of oleuropein and hydroxtyrosol. J. Pharm. Pharmacol. 51, 971–974.

Bongi, O., 1986. Oleuropein: an *Olea europaea* secoiridioid active on growth regulation. Acta Hort. 179, 245–249.

Brenes, M., Rejano, L., Garcia, P., Sanchez, A.H., Garrido, A., 1995. Biochemical changes in phenolic compounds during Spanish-style green olive processing. J. Agric. Food Chem. 43, 2702–2706.

Briante, R., Patumi, M., Limongelli, S., Ferdinando, F., Vaccaro, C., Di Salla, A., La Cara, F., Nucci, R., 2002. Changes in phenolic and enzymatic activities content during fruit ripening in two Italian Cultivars of *Olea europaea* L. Plant Sci. 162, 791–798.

Federici, E., Bongi, G., 1983. Improved method for isolation of bacterial inhibitors from oleuropein hydrolysis. Appl. Environ. Microbiol. 46, 509–510.

Fleming, H.P., Walter, W.M., Etchells, J.L., 1973. Antimicrobial properties of oleuropein and products of its hydrolysis from green olives. Appl. Microbiol. 26, 777–782.

Gutfinger, T., 1981. Polyphenols in olive oils. J. Am. Oil Chem. Soc. 58, 966–968.

Lavee, S., Avidan, N., 1982. Growth responses of tree callus to chlorogenic acid and related phenolic substances. In: Proceedings of the Fifth International Congress Plant Tissue and Cell Culture, Japan, pp. 165–168.

Lavee, S., Avidan, N., Pierik, R.L.M., 1994. Chlorogenic acid an independent morphogenesis regulator or a cofactor. Acta Hort. 381, 405–412.

Lo Scalzo, R., Scarpati, M.L., Verzengnassi, B., Vita, G., 1994. *Olea europaea* chemical repellent to *Dacus oleae* females. J. Chem. Ecol. 20, 1813–1823.

Malik, N.S.A., Bradford, J.M., 2005a. Design and construction of an inexpensive plexiglass chilling chamber to study flowering in olives. HortScience 40, 496–497.

Malik, N.S.A., Bradford, J.M., 2005b. Is chilling a prerequisite for flowering and fruiting in 'Arbequina' olives? Int. J. Fruit Sci 5, 29–39.

Malik, N.S.A., Bradford, J.M., 2005c. A simple protein extraction method for proteomic studies on olive leaves. J. Food Agric. Environ. 3, 246–248.

Manna, C., D'Angelo, S., Migliardi, V., Loffredi, E., Mazzoni, O., Morrica, P., Galletti, P., Zappia, V., 2002. Protective effect of phenolic fraction from

virgin olive oils against oxidative stress in human cells. J. Agric. Food Chem. 50, 6521–6526.

Owen, R.W., Giacosa, A., Hull, W.E., Haubner, R., Spiegelhalder, B., Bartsch, H., 2000. The antioxidant/anticancer potential of phenolic compounds isolated from olive oil. Eur. J. Cancer 36, 1235–1247.

Ozkaya, M.T., Celik, M., 1999. Quantitative analysis of phenolic compounds in olive cuttings. Acta Hort. 474, 477–480.

Romani, A., Mulinacci, N., Pinelli, P., Vincieri, F.F., Cimato, A., 1999. Ploy phenolic content in five Tuscany cultivars of *Olea europaea* L. J. Agric. Food Chem. 47, 967–969.

Ryan, D., Robards, K., Lavee, S., 1999. Changes in phenolic content of olive during maturation. Int. J. Food Sci. Technol. 34, 265–274.

Ryan, D., Prenzler, P.D., Lavee, S., Antolovich, M., Robards, K., 2003. Quantitative changes in phenolic content during physiological development of olive (*Olea europaea*) cultivar Hardy's Mammoth. J. Agric. Food Chem. 51, 2532–2538.

Soler-Rivas, C., Espin, J.C., Wichers, H.J., 2000. Oleuropein and related compounds. J. Sci. Food Agric. 80, 1013–1023.

Tripoli, E., Giammanco, M., Tabacchi, G., Di Majo, D., Giammanco, S., La Guardia, M., 2005. The phenolic compounds of olive oil: structure, biological activity and beneficial effects on human health. Nutr. Res. Rev. 18, 98–112.

Uccella, N., 2001. Olive biophenols: biomolecular characterization, distribution and phytolexin histochemical localization in the drupes. Trends Food Sci. Technol. 11, 315–327.

Visioli, F., Bellosta, S., Galli, C., 1998. Oleuropein, the bitter principle of olives, enhances nitric oxide production by mouse macrophages. Life Sci. 62, 541–546.

Wiseman, S.A., Mathot, J., De Fouw, N.J., Tijburg, L.B.M., 1996. Dietary non-tocopherol antioxidants present in extra virgin olive oil increase the resistance of low density lipoproteins to oxidation in rabbits. Atherosclerosis 120, 1–2.